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The steroid receptor RNA activator protein is recruited to promoter regions and acts as a transcriptional repressor

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ABSTRACT

Products of the steroid receptor RNA activator (SRA1) gene have the unusual property to function both at the RNA and the protein levels. SRA-RNA has long been known to increase the activity of multiple nuclear receptors. It has more recently been proposed than steroid receptor RNA activator protein (SRAP) also modulates steroid receptors activity. Herein, we show for the first time that SRAP physically interacts with multiple transcription factors and is recruited to specific promoter regions. Artificially recruiting SRAP to the promoter of a luciferase reporter gene under the control of the strong transcriptional activator VP16 leads to a decrease in transcription. Altogether we propose that SRAP could be a new transcriptional regulator, able to function as a repressor through direct association with promoters.

Structured summary:
MINT-7761068: *SRAP* (uniprotkb:Q9HD15) *physically interacts* (MI:0915) with *HDAC2* (uniprotkb:Q92769) by *anti bait coimmunoprecipitation* (MI:0006)

1. Introduction

The steroid receptor RNA activator (SRA) has originally been characterized as a non-coding transcript specifically co-activating steroid receptors [23,25]. This transcript acts embedded in ribonucleo-protein complexes containing steroid receptors as well as other proteins, such as the RNA helicase p68 or the SRA stem-loop interacting RNA binding protein (SLIRP), physically interacting with SRA and acting either as positive or negative functional regulators [2,9,25]. Secondary sub-structures (STRs) located within SRA core sequence (exons 2–5) are critical for these physical interactions to occur and therefore directly contribute to the co-activator role of this messenger [24]. It has now been demonstrated that SRA-RNA regulates the activity of additional transcription factors, including other nuclear receptors (such as the vitamin D (VDR) and the retinoic acid (RAR) receptors) and the myogenic differentiation factor MyoD [25]. This functional transcript therefore appears to have a much broader role than originally anticipated, participating in multiple normal and pathological events including tissue differentiation and tumorigenesis [2,9,25].

Coding SRA-RNAs, differing from the originally described non-coding RNA by an extended exon-1, have now been characterized [5,11,25]. This additional sequence contains two methionine codons, respectively, initiating a 236 and a 224 amino-acids long open reading frame terminated in exon-5. The corresponding endogenous proteins steroid receptor RNA activator proteins (SRAPs), that has been detected in multiple tissues and cell lines [6,22,34], contain two phylogenetically conserved domains (amino-acids 15–52 and amino-acids 135–204 [25]). This strong conservation suggests that both domains contribute to SRAP potential functions.

Most studies only focused on SRA-RNA and little is known about SRAP putative functions. It has nonetheless been proposed that SRAP, as its RNA counterpart, might also modulate the activity of steroid receptors [1,7,20,22]. SRAP was indeed found to physically interact with the androgen receptor and to enhance its ligand-induced transcriptional activity in prostate cells [20,22]. Transient

Abbreviations: SRA, steroid receptor RNA activator; SRAP, steroid receptor RNA activator protein; HDAC, histone de-acetylase; TSA, trichostatin A

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transfection experiments and reporter assays have further shown that SRAP could also enhance the transcriptional activities of estrogen (ESR1), glucocorticoid (GR) and peroxisome proliferator-activated receptor gamma (PPARγ) receptors [7,20,22]. Altogether, SRAP is currently suspected to act as positive regulator of several nuclear receptors.

The potential involvement of SRAP in mechanisms underlying transcriptional regulation is further substantiated by the identity of proteins characterized as co-immunoprecipitating with SRAP in Hela cells [19,25,28]. Known transcriptional regulators, such as SMARCC2 (member of the SWI/SNF chromatin remodeling complex, [35]) or the RNA helicase p68 [16], have indeed been identified as forming complexes with SRAP [19,28]. Most interestingly, the identification of the myocyte enhancer factor 2A (MEF2A) as one of SRAP protein partners further suggested that, beside androgen receptor, SRAP might also physically interact with other transcription factors. This would imply that SRAP, as its RNA, might modulate the transcriptional activity of a much wider range of transcription factors than initially predicted. Herein, we first used protein array to investigate SRAP potential interactions with multiple transcription factors and Chromatin immunoprecipitation (ChIP) to identify promoter regions potentially recruiting SRAP. We further assessed the potential effect of recruiting SRAP to the promoter region of an actively transcribed reporter gene.

2. Materials and methods

2.1. Plasmids

CMV-Renilla-luciferase, LexA-VP16, and L8G5-luciferase plasmids were previously described [3,6,17]. SDM1 and SDM7 mutations, impairing SRA-RNA action [24], were introduced in SRAP coding sequence using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) to generate SRAP-SDM as described [7]. GAL4-SRAP-SDM, GAL4-SRAP-N and GAL4-SRAP-C were obtained by, respectively, cloning full-length SRAP-SDM, aa 1–100, and aa 101–236 in frame with an existing GAL4 coding plasmid as described for the Receptor-Interacting Protein 140 [3].

2.2. Reporter assays

LexA-VP16 Luciferase reporter assays were performed using Hela cells as previously described [3,17]. Briefly, Hela cells were co-transfected with 0.1 μg of L8G5-luciferase, 0.05 μg LexA-VP16, 0.025 μg Renilla luciferase and 0.1 μg of GAL4, GAL4-SRAP-SDM, GAL4-SRAP-SDM-N, or GAL4-SRAP-SDM-C. Trichostatin A (TSA, Sigma, St. Louis, MO) was added to the indicated concentrations 16 h before lysis. Cells were harvested 24 h after transfection and Renilla luciferase and luciferase activities assessed and analyzed as previously described [3,17]. Following normalization to Renilla luciferase (accounting for transfection efficiencies), relative luciferase activity (RLU) of cells transfected with GAL4 alone with LexA-VP16 was arbitrarily assigned as 1. Luciferase activities within cells transfected with other vectors were expressed relatively. Results represent the average of at least three independent experiments performed in triplicate. Standard deviations were calculated and differences between results obtained with the various constructs and control (GAL4) were tested using the Student's *t*-test (two-tailed distribution, two-sample equal variance).

2.3. Immunoprecipitation and histone de-acetylase (HDAC) activity assay

Human breast cancer cell lines expressing SRAP-V5-tagged (MCF-7-SRAP-V5-High.A) and control MCF-7-SE cells were cultured as previously described [6]. Nuclear proteins were isolated (Panomics, Redwood City, CA) and incubated with agarose-beads-anti-V5 antibodies (Sigma, St. Louis, MO) according to the manufacturer's instructions to immunoprecipitate SRAP-V5 containing complexes. As an additional control, MCF-7-SRAP-V5-High.A extract was immunoprecipitated in the presence of V5 peptide (350 excess binding capacity of beads). HDAC activities were assayed using the fluorometric HDAC assay kit (Abcam, Cambridge, MA) as indicated by the manufacturer. For each experiment ($n = 4$) and each cell type, the immunoprecipitated HDAC activity was expressed as a percentage of the value before immunoprecipitation and normalized to the average residual background signal obtained with MCF-7-SE cells. Results correspond to the average immunoprecipitated HDAC activity expressed as percentage of total activity. Significant differences ($P < 0.05$) between samples were assessed using the Student's *t*-test (two-tailed distribution, paired).

Nuclear extracts from MCF-7 breast cancer cells were prepared and endogenous SRAP immunoprecipitated using a rabbit polyclonal anti-SRAP antibody (#A300-743A, Bethyl Laboratories Inc., Montgomery, TX) as described [11,33]. Irrelevant isotype matched antibodies (rabbit anti-goat IgGs, Jackson Immunoresearch Lab-Inc., West Grove, PA) were used as non-specific control. Presence of HDAC-2 in co-immunoprecipitated lysate was assessed by Western blot (Cell Signaling Technology Inc., Boston, MA) as previously reported [11,33].

2.4. Transcription factor protein array

Transcription factors protein-array analysis (TF-array, Panomics, Redwood City, CA) was performed as per manufacturers' instructions. Briefly, TF-array-I was incubated with 5 μg of recombinant SRAP protein (ProMab Biotechnologies Inc., Albany, CA) or without (control blot) for 2 h at room temperature. Immunodetection was performed using a primary anti-SRAP antibody (targeting aa 20–34) and chemiluminescence signals were captured and analyzed as described [6]. For both control and samples blots, intensity of the area (in counts/mm^2) encompassing the two spots corresponding to each transcription factor was first corrected by subtracting the signal of an immediately negative adjacent area (local background). For each transcription factor, the relative interaction (RI) was then determined by subtracting signal-control from signal-sample. Interactions were arbitrarily classified as strong (+++, RI > 15 000 counts/mm^2), positive (++, 1000 < RI < 10 000 - counts/mm^2), intermediate (+, 400 < RI < 1000 counts/mm^2) and weak/negative (RI < 400 counts/mm^2).

2.5. Isolation of DNA-associated proteins by formaldehyde cross-linking

DNA-associated proteins from MCF-7-SRAP-V5-High.A cells were isolated as previously described [31]. As a control for non-specific precipitation by hydroxy-apatite, not cross-linked cells extracts were used. Proteins precipitated with hydroxy-apatite were detected by Western blot using Anti-SRAP antibodies, anti-SP3 and anti-GAPDH antibody as described [6].

2.6. Chromatin immunoprecipitation assays and analysis

Chromatin immunoprecipitation assays were conducted as described [33]. Briefly, MCF-7 cells stably expressing SRAP-V5 cells were cultured in complete DMEM supplemented with 5% FBS. Cells were treated with formaldehyde in order to cross-link protein to DNA. Cells were then lysed and nuclear extracts were sonicated. The supernatants were then incubated overnight with a mouse anti-V5 antibody (Invitrogen) or the V5 antibody pre-incubated with the V5 peptide (negative control) (In a 100-fold excess). The immuno-complexes were collected by addition of protein

G-Sepharose (Amersham). The proteins were digested with proteinase K. DNA fragments were purified with a QIAquick Spin Kit (Qiagen, CA) and amplified by using the WGA amplification kit (Sigma, St. Louis, MO) according to the manufacturers protocol. The precipitated samples were sent to Nimblegen for Chromatin immunoprecipitation on CHIP (ChIP on CHIP) analysis using the human ChIP 385K Refseq promoter array (Nimblegen). Promoter regions that were enriched by at least 4-folds (peak score >2) in the SRAP-V5 precipitated sample compared to the negative control were further analyzed. Using the Genomatix RegionMiner software (Ann Arbor, MI), we determined the frequency of transcription factor binding sites and their over-representation against an average corresponding to the population of all annotated promoter regions. Obtained *Z*-score corresponds to the distance from the promoter population mean in units of the population standard deviation.

3. Results and discussion

3.1. SRAP interacts with multiple transcription factors

The fact that SRAP was found to directly interact with the androgen receptor [20] and form complexes with another known transcription factor MEF2A [19,28], led us to investigate whether it could also interact with other transcription factors. Using protein array (Fig. 1), we found that 29 out of 48 different transcription factors tested directly interacted with recombinant SRAP. The strength of these interactions varies upon the transcription factor considered. As such, SRAP appears to more strongly interact with ESR2 than with ESR1 (Table 1). This raises the interesting concept

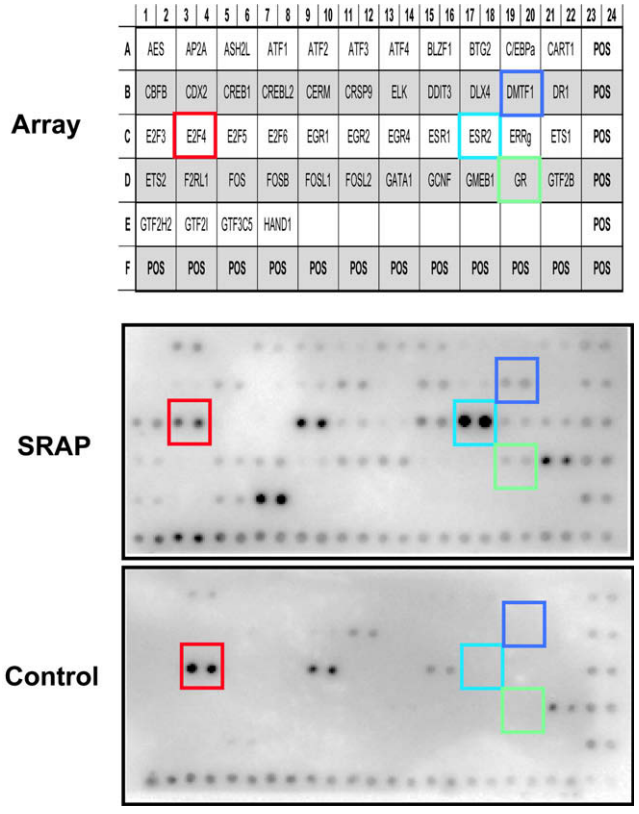

Fig. 1. SRAP directly interacts with transcription factors in vitro. A protein array containing 48 transcription factors spotted in duplicate was incubated with human recombinant SRAP (SRAP) or no protein (control) before immunodetection with anti-SRAP antibodies as outlined in Section 2. Following signal analysis, interactions with SRAP were qualified as high (+++, light blue box), positive (++, dark blue box), intermediate (+, green box) or weak/negative (–, red box). Relative interactions are detailed in Table 1.

that in specific contexts, the relative amount of estrogen receptor beta might interfere with the formation of complexes between SRAP and estrogen receptor alpha. This possibility might become of particular interest when considering the differential expression and roles played by these two receptors in breast tumorigenesis and tumor progression [8,30]. The observation that SRAP directly interacts with estrogen and glucocorticoid receptors fits with previous data from Kawashima et al. showing a direct interaction between SRAP and androgen receptor. This suggests that SRAP has the ability to modulate the action of steroid receptors (ESR1, AR and GR) likely through direct interactions.

Beside steroid receptors, several other transcription factors such as FOS [27], GATA1 [32] and ETS1 [10], known to participate in critical normal developmental steps or to events underlying tumorigenesis, directly interact with SRAP (Table 1). It is of interest that an interaction has been observed between SRAP and HAND1, essential to heart development [13]. Indeed, a recent study has

Table 1
Transcription factors analyzed for their direct physical interaction with SRAP.

Transcription factor description		Relative interaction[a]	
AES	Amino-terminal enhancer of split	61	–
AP2A	Transcription factor AP-2 alpha	2346	++
ASH2L	Ash2 (absent, small, or homeotic)-like	316	–
ATF1	Activating transcription factor 1	810	+
ATF2	Activating transcription factor 2	996	+
ATF3	Activating transcription factor 3	595	+
ATF4	Activating transcription factor 4	1158	++
BLZF1	Basic leucine zipper nuclear factor 1	965	+
BTG2	B-cell translocation gene 2	465	+
C/EBPa	CCAAT/enhancer binding protein alpha	739	+
CART1	Cartilage paired-class homeoprotein	840	+
CBFB	CBFB: core-binding factor, beta subunit	0	–
CDX2	CDX2: caudal type homeo box transcription factor 2	0	–
CERM	cAMP responsive element modulator	146	–
CREB1	cAMP responsive element binding protein 1	1051	++
CREBL2	cAMP responsive element binding protein-like	0	–
CRSP9	Cofactor-required for Sp1 transcriptional activation, subunit 9	0	–
DDIT3	DNA-damage-inducible transcript 3	2594	++
DLX4	Distal-less homeobox 4	172	–
DMTF1	Cyclin D binding myb-like transcription factor	2663	++
DR1	Down-regulator of transcription 1, TBP-binding	326	–
E2F3	E2F transcription factor 3	3286	++
E2F4	E2F transcription factor 4	0	–
E2F5	E2F transcription factor 5	0	–
E2F6	E2F transcription factor 6	0	–
EGR1	Early growth response 1	3373	++
EGR2	Early growth response 2	913	+
EGR4	Early growth response 4	398	–
ELK	ELK1, member of ETS oncogene family	0	–
ESR1	Estrogen-related receptor gamma	2805	++
ESR2	Estrogen receptor alpha	25 289	+++
ERRg	Estrogen receptor beta	1954	++
ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1	1607	++
ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2	435	+
F2RL1	Coagulation factor II (thrombin) receptor-like 1	0	–
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	1126	++
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	1468	++
FOSL1	FOS-like antigen 1	0	–
FOSL2	FOS-like antigen 2	2097	++
GATA1	GATA binding protein 1	2215	++
GCNF	Nuclear receptor subfamily 6, group A, member 1	125	–
GMEB1	Glucocorticoid modulatory element binding protein 1	0	–
GR	Glucocorticoid receptor	735	+
GTF2B	General transcription factor IIB	5478	++
GTF2H2	General transcription factor IIH, polypeptide 2	578	+
GTF2I	General transcription factor II, I	0	–
GTF3C5	General transcription factor IIIC, polypeptide 5	1195	++
HAND1	Heart and neural crest derivatives expressed 1	16 784	+++

[a] High (+++), positive (++), intermediate (+) and weak/negative (–) interaction.

shown that knocking down SRA gene led to myocardial contractile dysfunction in zebrafish, linking for the first time SRA and heart diseases [15]. The wide range of transcription factors identified as interacting with SRAP suggests that many normal and pathogenic events might involve this newly described protein.

In normal conditions, most transcription factors and nuclear receptors are localized and exert their functions in the nucleus. In breast cancer cells, SRAP, which contains a nuclear localization signal, is detected both in the cytoplasm and the nucleus [11,25,34]. The potential for this protein to localize in the nucleus, further demonstrated by the identification of SRAP and many of its co-immunoprecipitated partners in nuclear extracts from Hela cells [19,28], implies a potential role in this cellular compartment. Altogether, this led us to investigate the possibility of a recruitment of SRAP directly on chromatin.

3.2. SRAP is associated with chromatin

We used a previously described assay consisting in cross-linking DNA and associated proteins, precipitating DNA, and analyzing co-precipitated proteins using Western blot [31]. We found that both exogenous tagged and endogenous SRAP precipitated with DNA in cells stably expressing SRAP-V5 tagged protein (MCF-7-SRAP-V5-High.A cells [6], Supplementary material, Fig. S1). As anticipated, both long (SP3L) and short (SP3M) transcription factor SP3 isoforms, known to be associated with DNA, were also detected in the DNA bound protein fraction. None of these associations were observed in the absence of cross-linking. On the other hand, the mainly cytoplasmic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was present in the protein lysate but not associated with DNA. The fact that SRAP could indeed be associated with chromatin led us to investigate further whether SRAP might be associated to specific promoter regions.

3.3. SRAP binds to promoter regions enriched in nuclear receptor binding sites

To identify promoter regions potentially recruiting SRAP, we performed Chromatin immunoprecipitation (ChIP)-CHIP arrays. Breast cancer cells stably expressing SRAP-V5 tagged protein [6] were treated, DNA precipitated with anti-V5 antibody, and recognized promoter regions identified as described in Section 2. As a negative control, we used a V5 antibody pre-incubated with 100-fold excess V5 peptide. We were able to identify 2319 regions that were enriched at least by four folds in the SRAP-V5 chromatin immunoprecipitated samples as compared to the negative control (Supplementary material, S2). The transcription factor binding sites present within these sequences have been sorted according to their over-representation coefficient (*Z*-score). A list of the name of transcription factor families corresponding to the 40 most over-represented binding sites out of the 176 present is shown Table 2. Binding sites for nuclear receptors (in italics) are highly enriched in

Table 2
Partial list of transcription factor (TF) binding sites families found in 2319 sequences identified following ChIP-CHIP array.

Family of TF	Number of sequences	Number of matches	*Z*-Score[a]	Transcription factor families full name	Rank/176
V$NR2F	*1652*	*3300*	*11.33*	*Nuclear receptor subfamily 2 factors*	*1*
V$GREF	*1057*	*1856*	*9*	*Glucocorticoid responsive and related factors*	*2*
V$PERO	*920*	*1218*	*8.39*	*Peroxisome proliferator-activated receptor*	*3*
V$SRFF	886	1583	8.34	Serum response element binding factor	4
V$STAT	1265	2843	7.13	Signal transducer and activator of transcription	5
V$CAAT	1163	1766	6.89	CCAAT binding factors	6
V$AIRE	358	408	6.87	Autoimmune regulatory element binding factors	7
V$RBPF	688	869	6.76	RBPJ – kappa	8
V$IKRS	838	1107	6.72	Ikaros zinc finger family	9
V$HAML	648	792	6.62	Human acute myelogenous leukemia factors	10
V$GATA	**1401**	**2608**	**6.51**	**GATA binding factors**	**11**
V$EREF	*685*	*936*	*6.38*	*Estrogen response elements*	*12*
V$MOKF	879	1147	6.24	Mouse Krueppel like factor	13
V$TALE	840	1140	5.98	TALE homeodomain class recognizing TG motifs	14
V$PRDF	753	994	5.54	Positive regulatory domain I binding factor	15
V$ZFTR	561	683	5.4	Zinc finger transcriptional repressor	16
V$YY1F	1109	1618	5.34	Activator/repressor binding to transcription initiation site	17
V$SORY	1629	3905	5.33	SOX/SRY-sex/testis determining and related HMG box factors	18
V$SF1F	565	683	5.22	Vertebrate steroidogenic factor	19
V$NBRE	*394*	*437*	*5.19*	*NGFI-B response elements, nur subfamily of nuclear receptors*	*20*
V$RXRF	*1640*	*3386*	*5.13*	*RXR heterodimer binding sites*	*21*
V$LEFF	801	1043	5.12	LEF1/TCF	22
V$BTBF	307	320	5.06	BTB/POZ transcription factor	23
V$RP58	324	371	5.06	RP58 (ZFP238) zinc finger protein	24
V$CLOX	1099	2055	5.02	CLOX and CLOX homology (CDP) factors	25
V$EVI1	1455	3101	4.98	EVI1-myleoid transforming protein	26
V$TEAF	645	761	4.94	TEA/ATTS DNA binding domain factors	27
V$HEAT	1142	1937	4.79	Heat shock factors	28
V$BCDF	953	1510	4.67	Bicoid-like homeodomain transcription factors	29
V$HAND	**1433**	**2861**	**4.64**	**Twist subfamily of class B bHLH transcription factors**	**30**
V$HMTB	610	763	4.6	Human muscle-specific Mt binding site	31
V$HOXH	750	1009	4.6	HOX – MEIS1 heterodimers	32
V$NEUR	891	1223	4.46	NeuroD, Beta2, HLH domain	33
V$SIXF	543	663	4.39	Sine oculis (SIX) homeodomain factors	34
V$SMAD	610	777	4.34	Vertebrate SMAD family of transcription factors	35
V$PBXC	721	936	4.34	PBX1 – MEIS1 complexes	36
V$GCMF	601	763	4.32	Chorion-specific transcription factors with a GCM DNA bind. domain	37
V$AP1F	**548**	**872**	**4.31**	**AP1, Activating protein 1**	**38**
V$PAX8	598	728	4.3	PAX-2/5/8 binding sites	39
O$INRE	636	794	4.29	Core promoter initiator elements	40

[a] Distance from the population mean in units of the population standard deviation.

promoter sequences precipitating with SRAP. These include nuclear receptor subfamily two factors binding element, as well glucocorticoid responsive, peroxisome proliferator-activator receptor and estrogen receptor responsive elements. These results suggest that previously seen physical interactions between nuclear receptors such as GR or ESRs and SRAP might result in the targeting of this later protein to specific promoter regions. Interestingly, binding sites for GATA, HAND and AP1 (recognized by FOS) are also enriched (Table 2 in bold). This in turn strongly suggests that SRAP might also be recruited to particular promoter regions through physical interaction with transcription factors such as GATA1, HAND1 or FOS. This is the first time SRAP is found recruited at promoter levels. The overall effect of recruiting SRAP on a given promoter region is likely to be specific of the exact region involved, the existing genomic context, the gene considered, as well as the presence or absence of other factors. Individual studies are therefore obviously needed to decipher the exact role SRAP might have on the transcription of genes controlled by promoters it is located on. It was however of interest to investigate whether forcing the recruitment of SRAP on a given promoter might result in any transcriptional modification.

3.4. SRAP has a transcriptional repressive activity sensitive to TSA

In order to determine the generic effect of recruiting SRAP at a given promoter, we used an artificial luciferase reporter system to recruit the hybrid GAL4-SRAP protein in close proximity to the LEXA-VP16 transcriptional activator on the promoter of the GAL4-LEXA-luciferase reporter vector. To exclude any potential influence of SRA-RNA in the effect observed, we used a construct (GAL4-SRAP-SDM), which contains silent mutations (SDM1 and SDM7) shown to alter sub-structures STR1 and 7 and interfere with SRA-RNA co-activation function [24]. We first analyzed the ability of full-length SRAP-SDM to modulate the activity of LexA-VP16 when physically recruited on adjacent promoter sequences (Fig. 2A and B). We found that GAL4-SRAP-SDM decreased the

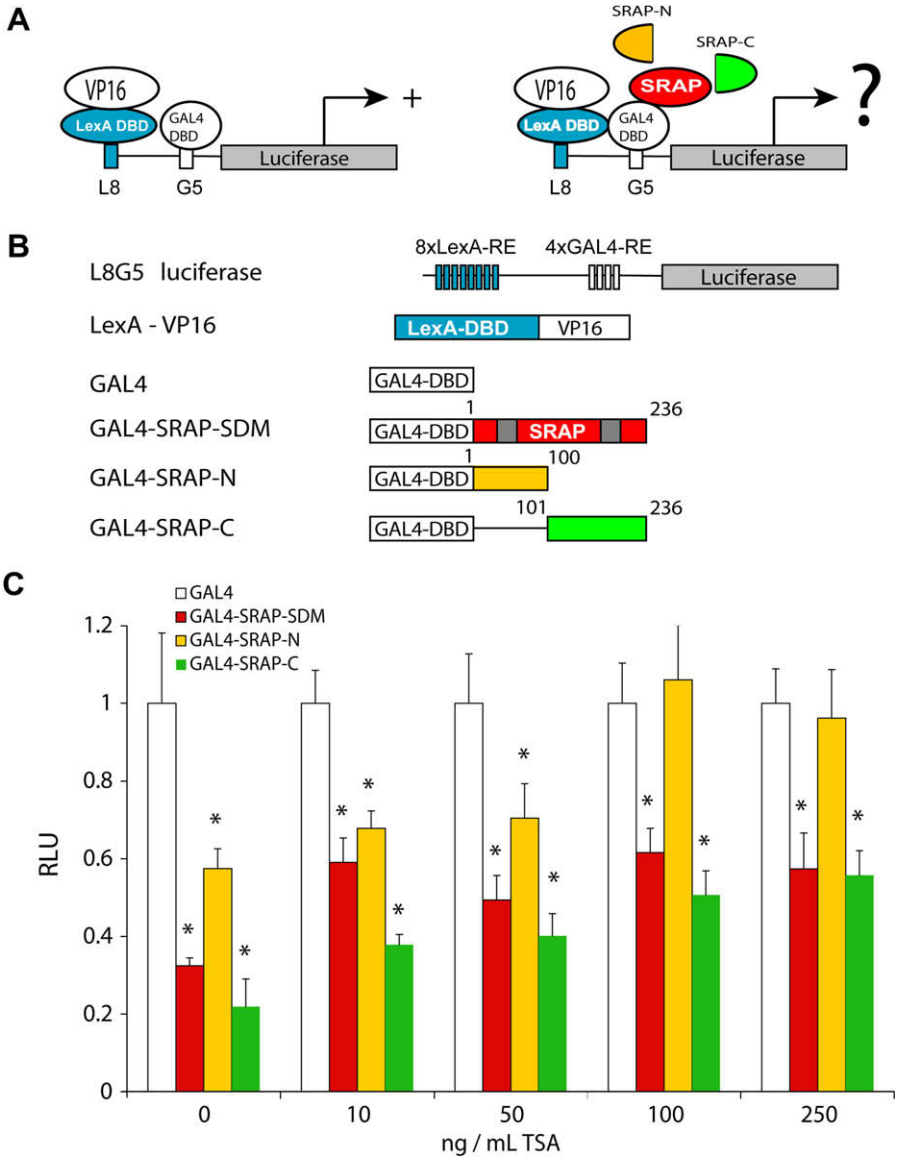

Fig. 2. SRAP and its two conserved domains repress transcription when physically recruited at the promoter level. (A) Schematic of LexA-VP16 assay. (B) Constructs used. (C) Hela cells were co-transfected with L8G5-luciferase together with GAL-4, GAL4-SRAP-SDM, GAL4-SRAP-N or GAL4-SRAP-C, treated with increasing amount of TSA and luciferase activity assessed as described in Section 2. Bars and stars represent standard deviations ($n = 4$) and significant (Student's t-test, $P < 0.05$) difference with GAL4 control, respectively.

activity of VP16 in this system (Fig. 2C). It should be noted that a construct encoding for SRAP-SDM but exempt of GAL4-DBD (therefore not physically recruited to the promoter) did not have any effects in this system (data not shown). As outlined earlier, SRAP contains two N- and C-terminal phylogenetically conserved domains suspected to participate in SRAP functions. When analyzing the effect of recruiting these domains, we found that the C-terminal domain had by itself an inhibitory effect similar to the full-length molecule (~70–80% inhibition) whereas the N-terminal region, also acting as a repressor, had a weaker impact (40% inhibition). Treatment with TSA (an inhibitor of HDAC activity) fully abolished the repressive activity of the N-terminal domain (0% inhibition of VP16 activity at 100 ng/mL TSA) but only partially inhibited the effect of the full-length protein or its C-terminal domain (~40% inhibition). The differential impact of TSA treatments on the respective repressive action of these domains underlines a possible heterogeneity of the mechanisms involved. It indeed suggests that while the N-terminal mechanism of action mainly involves HDACs, the C-terminal conserved domain might recruit additional inhibitory proteins insensitive to TSA treatment. To further investigate whether SRAP action might potentially involve the recruitment of HDAC activity, we assessed HDAC activity in SRAP-V5-tagged protein co-immunoprecipitated nuclear extracts from previously described breast cancer cells (MCF-7-SRAP-V5-High.A) stably expressing this protein (Fig. 3A). As control of non-specifically immunoprecipitated HDAC activity we treated in parallel extracts from non-expressing cells (MCF-7-SE). We found that 0.2% of total nuclear HDAC activity specifically co-immunoprecipitated with SRAP-V5. To confirm that endogenous SRAP could also form complexes with known molecules harboring HDAC activity, we have performed co-immunoprecipitation experiments using untransfected MCF-7 breast cancer cell line nuclear extracts. We found that HDAC-2 was associated with endogenous SRAP in this model (Fig. 3B).

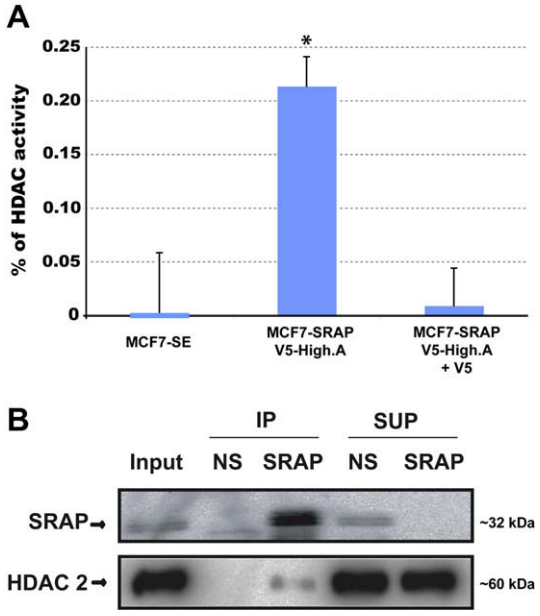

Fig. 3. Specific co-immunoprecipitation of SRAP-V5 and HDAC activity. (A) Nuclear extracts from MCF-7-SE (control) and MCF-7-SRAP-V5-High.A cells were immunoprecipitated with anti-V5 antibodies and HDAC activity measured as detailed in Section 2. MCF-7-SRAP-V5-High.A extract was also treated in parallel with competitive V5 peptide. Standard errors ($n = 4$) and significant (Student's t-test, $P < 0.05$) difference with MCF-7-SE (control) are indicated by bars and stars, respectively. (B) Nuclear extract from MCF-7 cells were divided into two pools and subsequently immunoprecipitated with rabbit polyclonal anti-SRAP antibodies (SRAP) or with non-specific rabbit polyclonal antibodies (NS) as described in Section 2. Immunoprecipitated fractions (IP) or supernatant (Sup) were checked by Western blot using anti-SRAP and anti-HDAC-2 antibodies. Nuclear extracts (Input) was used as positive control.

The potential to act through both HDACs and non-HDAC dependent mechanisms has been demonstrated for other transcription repressors. For example, the short heterodimer partner (SHP) and the ligand-dependent nuclear receptor co-repressor LcoR can act through EID1 (EP300 interacting inhibitor of differentiation 1) and CtBP (C-terminal binding protein), respectively [12,17]. The identity of the non-HDAC proteins possibly involved in SRAP mediated transcription repression remains to be determined.

The present observation that SRAP has an intrinsic repressive ability contrasts with previous results presenting SRAP as an activator of transcriptional activity [1,7,20,22]. It should however be noted that transcriptional co-regulators are known to behave differentially in different cellular and promoter contexts. For example, SRA differentially activates estrogen receptor controlled transcription of reporter genes driven by different estrogen receptor elements [21]. Similarly, molecules such as the co-activator independent of AF-2 function (CIA) or the zinc-finger gene involved in apoptosis and cell-cycle control (ZAC1), have the ability to act either as co-activator or co-repressor [18,29]. Further studies are urgently needed to establish what are the exact effects of SRAP on the transcription of specific genes controlled by a particular transcription factor in a given context.

The binding of a given transcription factor on a specific promoter results in the sequential recruitment of multiple co-regulatory molecules. Using ChIP-re-ChIP experiments, Metivier et al. have elegantly illustrated the complexity of the dynamic events that occur following the initial recruitment of the estrogen receptor alpha on the promoter of the estrogen dependent pS2 gene [26]. Similar experiments could be performed to characterize the co-recruitment of the estrogen receptor and SRAP on a given estrogen regulated promoter region. The two faces of the products of the SRA1 gene, a functional RNA and a protein, however make the choice of specific targets to be analyzed potentially challenging. Indeed, even though both SRA-RNA and SRAP are believed to regulate estrogen receptor activity [1,7,21,23], silencing SRA1 gene does not affect the induction of pS2 gene by estrogen [4]. More recently, Foulds et al. even reported that knocking down the expression of both RNA and protein only affected a very small subset of direct estrogen receptor target genes in MCF-7 breast cancer cells [14]. This emphasizes the need to first identify endogenous target genes specifically regulated by SRAP in order to further establish the biological significance of the binding on promoter sites of this protein, alone or associated with specific transcription factors. This could potentially be achieved through the use of specific models allowing the distinction between SRA-RNA and SRAP respective functions.

Interestingly, Foulds's study also demonstrated that even though silencing SRA modified the expression of a common subset of genes in Hela uterus and MCF-7 breast cancer cell lines, some other genes were differentially affected in the two cell types [14]. This highlights cell type differences in SRA/SRAP potential functions, potentially resulting from the different relative amount of transcription factors and co-regulators interacting with these two regulatory molecules. For a given gene, SRAP effects on transcription might therefore be positive or negative potentially as a direct result of the balance between multiple transcription factors present in specific cell types.

SRA is a very peculiar example of a bi-faceted system consisting of a functional RNA and its corresponding protein. Our results show for the first time that SRAP can physically interact with multiple transcription factors and is recruited by promoter regions. Altogether our data suggest that SRAP, as its RNA, has the potential to be involved in many critical pathways and putatively directly participates to the regulation of gene expression. Interestingly both

SRA-RNA and protein might be implicated in similar signaling pathways. Specific studies deciphering SRAP and SRA exact mechanisms of action in the context of particular transcription factors are warranted. Identifying SRA/SRAP target genes is crucial for a comprehensive understanding of the bi-faceted system represented by the products of this peculiar gene.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.04.022.

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